AEM
Journals.ASM.org

Environmental Factors Influencing the Prevalence of a *Clostridium botulinum* Type C/D Mosaic Strain in Nonpermanent Mediterranean Wetlands

Dolors Vidal,[a,b] Ibone Anza,[a] Mark A. Taggart,[a,c] Elisa Pérez-Ramírez,[a] Elena Crespo,[d] Ursula Hofle,[a] Rafael Mateo[a]

Instituto de Investigación en Recursos Cinegéticos (IREC), CSIC-UCLM-JCCM, Ciudad Real, Spain[a]; Departamento de Ciencias Médicas, Facultad de Medicina, Universidad de Castilla-La Mancha, Ciudad Real, Spain[b]; Environmental Research Institute, University of the Highlands and Islands, Thurso, Caithness, Scotland, United Kingdom[c]; Centro de Recuperación de Fauna El Chaparrillo, Ciudad Real, Spain[d]

Between 1978 and 2008, 13 avian botulism outbreaks were recorded in the wetlands of Mancha Húmeda (central Spain). These outbreaks caused the deaths of around 20,000 birds from over 50 species, including globally endangered white-headed ducks (*Oxyura leucoceophala*). Here, a significant association was found between the number of dead birds recorded in each botulism outbreak and the mean temperature in July (always >26°C). The presence of *Clostridium botulinum* type C/D in wetland sediments was detected by real-time PCR (quantitative PCR [qPCR]) in 5.8% of 207 samples collected between 2005 and 2008. Low concentrations of Cl^- and high organic matter content in sediments were significantly associated with the presence of *C. botulinum*. Seventy-five digestive tracts of birds found dead during botulism outbreaks were analyzed; *C. botulinum* was present in 38.7% of them. The prevalence of *C. botulinum* was 18.2% ($n = 22$ pools) in aquatic invertebrates (Chironomidae and Corixidae families) and 33.3% ($n = 18$ pools) in necrophagous invertebrates (Sarcophagidae and Calliphoridae families), including two pools of adult necrophagous flies collected around bird carcasses. The presence of the bacteria in the adult fly form opens up new perspectives in the epidemiology of avian botulism, since these flies may be transporting *C. botulinum* from one carcass to another.

Botulism poisoning is caused by the ingestion of a potent neurotoxin (botulinum neurotoxin [BoNT]) produced by *Clostridium botulinum* that produces flaccid paralysis and death. Seven toxin types have been designated (types A to G), with type C most frequently involved in cases of avian botulism (1, 2), followed by types D and E. Avian botulism has been diagnosed around the world (except in the Antarctic) and is considered the most important avian disease in terms of mortality (3). Recently, some cases of botulism in animals in Europe and Japan were caused by mosaics of type C and D toxins, for which a higher lethal activity is observed in mouse in comparison to other types of BoNT (4–6). This mosaic C/D type toxin seems to be predominant in European waterfowl and cross-reacts against type C antisera in the commonly used mouse bioassay (7).

Clostridium botulinum type C is not considered overtly pathogenic, but it acts as a saprophytic bacteria that uses a neurotoxin (BoNT) to kill in order to create an appropriate medium for its maintenance (8). The exponential mortality observed during outbreaks of avian botulism has been associated with the life cycle of necrophagous flies and their maggots. The maggots act as a carrier of BoNT from decomposing bird carcasses to live birds (9–16). In terms of botulism outbreaks, there are several predisposing factors which have complex relationships (3). One of these factors is the abundance of *C. botulinum* spores in the environment, which may in turn depend on local soil, sediment, and water properties (17–24). The elevated temperatures in wetlands that are reached mainly in summer can favor the growth of *C. botulinum* in carcasses or in decomposing organic material (25–27). Bird mortality due to other causes can contribute to botulism by providing carcasses where *C. botulinum* can grow and initiate an outbreak (28–30). Finally, the susceptibility of certain bird species or individuals to the BoNT toxin may be an important determinant (2, 31, 32).

Botulism can be a significant risk for endangered waterbird species, especially for those with populations concentrated in just a few wetlands or on islands, where an outbreak may reduce their numbers dramatically (33). White-headed duck (*Oxyura leucocephala*) and marbled teal (*Marmaronetta angustirrostris*) are endangered and vulnerable (34) waterfowl species, and southern Spain is one of their population strongholds within the western Palearctic region (35). Some of the wetlands used by these species as breeding sites in Spain, such as El Hondo on the Mediterranean coast, La Mancha Húmeda in central Spain and the Guadalquivir Marshes in southern Spain, can be considered areas where avian botulism is endemic, since outbreaks occur there almost every summer (22, 36, 37). Botulism may therefore continue to drive these already vulnerable species toward a more critical status and reduce the efficacy of important conservation efforts made over recent decades (38).

Here, we compiled available data on botulism outbreaks in the wetlands of La Mancha Húmeda in Spain for the last 20 years and explored their association with meteorological data. The presence of *C. botulinum* type C in wetland sediments was assessed using real-time PCR (quantitative PCR [qPCR]) and the relationship between its occurrence and the physicochemical characteristics of

Received 16 April 2013 Accepted 29 April 2013
Published ahead of print 3 May 2013
Address correspondence to Dolors Vidal, mariadolors.vidal@uclm.es.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.01191-13.

doi:10.1128/AEM.01191-13

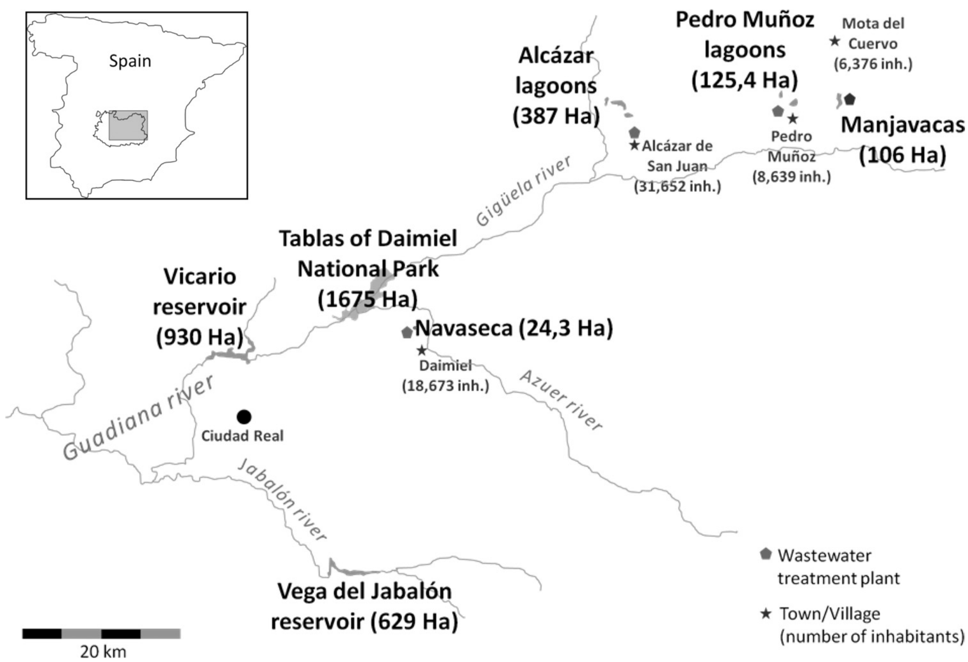

FIG 1 Locations of the studied wetlands in Castilla-La Mancha (central Spain). Most sites were located in the province of Ciudad Real; only Manjavacas was located in Cuenca. The surface areas (in hectares) are shown in parentheses on the map. inh, inhabitants.

the sediments analyzed. Moreover, the presence of *C. botulinum* in aquatic invertebrates and necrophagous flies sampled during botulism outbreaks was studied to evaluate their role in the epidemiology of these episodes.

MATERIALS AND METHODS

Study area. The study area consisted of wetlands in Castilla-La Mancha (central Spain), and the study focused on the National Park of Tablas de Daimiel and nearby wetlands where botulism outbreaks have been recorded since 1978 (Fig. 1). Tablas de Daimiel was declared a National Park in 1973 and a Special Protection Area for Birds in 1979 and was included in the Ramsar List in 1982. The National Park now protects the remaining 1,675 ha of a wetland that 50 years ago comprised 6,000 ha (39). Four of the wetlands studied also receive inputs from wastewater treatment plants from nearby towns/villages (Fig. 1). We included two reservoirs, because birds from the natural wetlands often use these sites for feeding or resting, since there are large areas of shallow water with abundant vegetation present (Fig. 1). The climate in this area is cold-temperate continental, with a pronounced dry season and annual rainfall of around 400 to 500 mm. All the lagoons studied are between 603 and 670 m above sea level (39). Manjavacas, Alcázar de San Juan, and Pedro Muñoz lagoons are characterized by a salt concentration of >5 g/liter even at the height of flooding, and they have historically shown marked seasonality in terms of water level (since water supplies to these lagoons were originally limited only to rainfall and runoff). However, water discharged from wastewater treatment plants into these lagoons has altered their natural hydrological character (40). Navaseca lagoon is a highly eutrophic artificial wetland which is close to Tablas de Daimiel Park (6.5 km away).

Historic data and sample collection. Waterfowl mortalities due to botulism outbreaks were compiled from official data recorded between 1978 and 2008 by the regional government (Junta de Comunidades de Castilla-La Mancha [JCCM]). The diagnosis of avian botulism was based on clinical observations made by veterinary staff at the Wildlife Rehabilitation Centres of JCCM and confirmed by mouse bioassay with a hexavalent antitoxin provided by the Centers for Disease Control and Prevention, Atlanta, GA, USA, and undertaken at the Spanish National Institute of Toxicology. The mouse bioassay was performed using sera from eight birds from five different outbreaks that occurred in Mancha Húmeda, Spain, between 1998 and 2002.

Monthly meteorological data (minimum, maximum, and mean temperatures, number of days with a temperature of >25°C, mean rainfall, and mean humidity) for the study area between 1997 and 2008 were obtained from the Spanish National Institute of Statistics (http://www.ine.es).

A total of 207 sediment samples were collected between 2005 and 2008 in the Spanish wetlands studied. In July 2005, sediment samples were collected from Tablas de Daimiel ($n = 14$), Alcázar de San Juan ($n = 24$), Pedro Muñoz ($n = 15$), Manjavacas ($n = 9$), Vicario ($n = 8$), and Vega del Jabalón ($n = 2$). In January 2006, sediment samples were collected from Tablas de Daimiel ($n = 11$), Vega del Jabalón ($n = 9$), and Vicario ($n = 10$). Additionally, between 2006 and 2008, sediment samples were collected in wetlands where avian mortalities had been detected during the summer, i.e., Tablas de Daimiel in 2007 ($n = 68$), Alcázar de San Juan in 2006 to 2008 ($n = 13$), Navaseca in 2008 ($n = 14$), and Jabalón in 2008 ($n = 2$). Sediment samples (50 to 100 g) were collected from the upper 0 to 5 cm. Benthic invertebrates, mostly larvae of members of the Chironomidae family (nonbiting midges), and water column invertebrates, mostly Corixidae (water bugs), were collected during sediment sampling and processed in pools grouped by sampling site.

Carcasses of 75 birds from 18 species were sampled during avian mortalities detected in the wetlands studied. These samples included gastric contents, samples from the intestine, cecum, and cloacal swabs, although not all could be taken from each bird. Additionally, 5 pools of adult necrophagous flies, mostly members of the Calliphoridae and Sarcophagidae families, were caught flying around bird carcasses, as were 10 pools of larvae, 3 pools of eggs, and 1 pool of pupae (of Calliphoridae), collected directly from bird carcasses. All samples were frozen immediately at −30°C and stored until analysis.

Detection of *C. botulinum* by preenrichment cultures and qPCR and PCR for type C/D. All samples were processed as previously described by Vidal et al. (37). Detection followed a protocol that included preenrichment of the sample by culture, DNA extraction, and detection of the gene encoding the toxin by qPCR. Cultivation of samples was performed

TABLE 1 Official mortality rates of birds due to avian botulism outbreaks in Castilla-La Mancha wetlands in Spain between 1978 and 2008

Family	No. of species	Mortality rate in the following yr:								Mortality rate for 1978 to 2008
		1978	1998	1999	2002	2004	2005	2006	2008	
Podicipedidae	4	83	2	10	0	1	0	3	0	99
Ardeidae	1	18	0	344	0	12	41	3	0	418
Ciconiidae	1	1	0	3	1	4	4	9	0	22
Phoenicopteridae	1	0	0	0	3	2	0	0	0	5
Anatidae	12	2,755	251	9,572	1	93	454	489	172	13,787
Accipitridae	2	4	0	0	0	0	0	2	0	6
Phasianidae	1	1	0	0	0	0	0	0	0	1
Rallidae	3	348	603	850	3	34	41	112	19	2,010
Recurvirostridae	2	513	191	38	11	15	6	29	2	805
Charadridae	4	577	0	7	6	1	1	2	1	595
Scolopaciae	12	1,081	0	21	0	4	0	0	10	1,116
Glareolidae	1	0	0	0	0	0	0	1	0	1
Laridae	3	316	343	117	16	4	0	65	2	863
Sternidae	6	64	0	18	1	0	1	60	0	144
Tytonidae	1	1	0	0	0	0	0	0	0	1
Laniidae	1	1	0	0	0	0	0	0	0	1
Passeriformes	1	0	0	0	0	0	0	4	0	4
Columbidae	1	0	0	0	0	0	0	1	0	1
Total	57	5,763	1,390	10,980	42	170	548	780	206	19,879

in a commercial cooked meat broth supplemented with vitamin K_1, glucose, and hemin (BD BBL cooked meat medium with glucose, hemin, and vitamin K; BD, NJ, USA) using an anaerobe container system (BD GasPak EZ; BD, NJ, USA) over a period of 3 to 5 days at 40°C. DNA extraction was performed using two commercial kits for DNA extraction, the PowerSoil DNA isolation kit (MoBio, Carlsbad, CA, USA) and the DNeasy blood and tissue kit (Qiagen, Hilden, Germany) for sediment samples and animal tissue samples, respectively (following the manufacturer's recommendations). qPCR was performed as described previously (41), including genes encoding both type C and type C/D mosaic toxins, and has been adapted for environmental samples by Vidal et al. (37). The amplicon examined by qPCR is within the amino-terminal domain of the heavy chain, and it specifically addresses position 2014 bp for the forward primer and 2112 bp for the reverse primer using the BoNT sequence of the type C/D mosaic strain 03-009 (GenBank accession number AB200360) (4). In order to confirm whether field samples were positive for type C/D mosaic, a total of 30 samples were also tested by a standard PCR by the method of Takeda et al. (4). The sensitivity of this PCR was compared to the sensitivity of qPCR using an isolated strain from the gastric contents of a black-headed gull (*Chroicocephalus ridibundus*), collected in the summer of 2005 in an outbreak that occurred in Alcázar de San Juan lagoon (internal reference IREC-B136) which was quantified by the most probable number (MPN) technique.

Determination of the physicochemical characteristics of the sediments. Each fresh sediment sample (10 g) was mixed with 30 ml of deionized water for 30 min on a shaker. The pH was measured in the solution after 2 min. The solution was vacuum filtered through 0.45-μm filter paper. This filtrate was then used to determine water-soluble PO_4^-, NO_3^-, NO_2^-, and Cl^- by means of UV-visible (UV-Vis) spectrophotometry using Sprectroquant kits (Merck, Darmstadt, Germany). Moisture and organic matter content were determined sequentially, first by drying the sample at 120°C in an oven to calculate the amount of free and combined water present in the sample and then by heating in a muffle furnace at 450°C to calculate the percentage loss on ignition (% LOI).

Statistical analysis. The relationship between avian mortality rate (due to botulism outbreaks) and meteorological data was analyzed with Spearman's correlation coefficient (r_s). The frequency of detection of *C. botulinum* in different types of samples was compared by the chi-square test or Fisher exact probability test. Physicochemical characteristics of sediments were compared among wetlands with one-way analysis of variance (ANOVA) tests. *Post hoc* differences were studied with Tukey tests. The association between the presence of *C. botulinum* and the physicochemical characteristics of the sediments was studied with generalized linear models (GLM) with a binary logistic distribution. The presence of *C. botulinum* (negative or positive) was used as the dependent variable, and the physicochemical characteristics of the sediments were used as the predictors. Physicochemical data were log transformed when necessary to approach a normal distribution. The models studied initially included all the known predictors, but only those with higher significance were retained in the final models following a backward stepwise procedure. Significance for the statistical analyses was set at $P \leq 0.05$, and analyses were performed with IBM SPSS Statistics 19.0.0.

RESULTS

Description of avian botulism outbreaks. Between 1978 and 2008, 13 botulism outbreaks were recorded within the study area. Around 20,000 individuals from >50 species from 18 families (Table 1; see Table S1 in the supplemental material) were found dead by environmental authorities during these outbreaks. The most frequently affected family was Anatidae, followed in smaller numbers by Rallidae and Scolopacidae (Table 1). In terms of species, the highest mortality rates were reported for mallards (*Anas platyrhynchos*), Eurasian coot (*Fulica atra*), gadwall (*Anas strepera*), and Northern shoveler (*Anas clypeata*) (Table S1). Two threatened waterfowl species (34), the endangered white-headed duck (*Oxyura leucocephala*) and the near-threatened ferruginous duck (*Aythya nyroca*) were also found dead in small numbers during these outbreaks. The presence of BoNT was confirmed by mouse bioassay in seven birds (six mallards [*Anas platyrhynchos*] and one common teal [*Anas crecca*]) collected in four different outbreaks prior to 2002. One negative sample was from a black-headed gull (*Chroicocephalus ridibundus*), which was sampled during recovery and finally released.

A significant association was found between the number of dead birds counted during the botulism outbreak and the mean temperature in July ($r_s = 0.745$; $P = 0.005$). Most of these out-

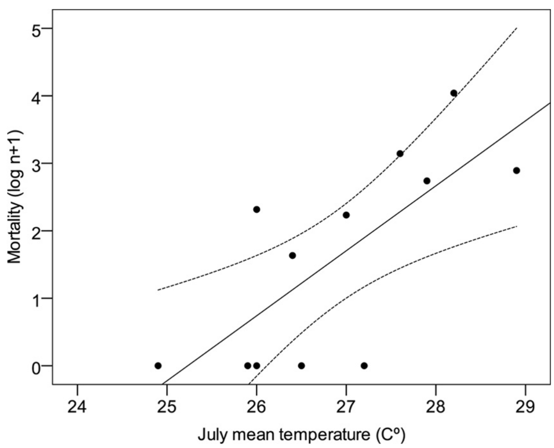

FIG 2 Relationship between avian mortalities (log transformed data) detected in botulism outbreaks and the mean temperature during the month of July between 1997 and 2008 ($r_s = 0.745$; $P = 0.005$). Shown is the regression (solid line) with 95% confidence interval (broken lines).

breaks (9 out of 13) were initiated or occurred in July, and all occurred when the mean temperature during this month was above 26°C (Fig. 2). No other correlations were found between botulism mortality and the rest of the meteorological data.

Identification of *C. botulinum* type. The strain isolated from one black-headed gull was confirmed as the type C/D mosaic by conventional PCR and qPCR. The sensitivity of qPCR with this strain was around 2.5 spores/ml (1 to 6 spores, 95% confidence interval), whereas for e conventional PCR, it was about 250 spores/ml (see Table S4 and Fig. S1 in the supplemental material). The presence of the type C/D mosaic was also confirmed by conventional PCR in 16 of 22 field samples that were previously positive to the qPCR.

***C. botulinum* in environmental samples and birds.** The overall prevalence of *C. botulinum* type C/D in preenriched sediment samples was 5.8%. The Alcázar de San Juan wetland showed the highest prevalence, followed by Vega del Jabalón and Tablas de Daimiel wetlands, with values of 10.8%, 9.5%, and 6.5%, respectively (Table 2). Most of the positive sediments (10 out of 12) were collected during botulism outbreaks, which always started in June or July (see Table S2 in the supplemental material). Interestingly, none of the 14 sediment samples collected during the Navaseca outbreak, which started at the end of September 2008, were positive by qPCR (Table 2).

All the physicochemical characteristics for the sediments were found to differ among wetlands (Table 3), and the characteristics that were significantly associated with the presence of toxigenic *C. botulinum* type C/D were lower Cl^- (Wald's $\chi^2 = 4.54$; $P = 0.033$) and higher %LOI (Wald's $\chi^2 = 5.08$; $P = 0.024$). These effects were observed in the final model which included %LOI, Cl^-, moisture, pH, and NO_3^-. The effect of Cl^- was maintained across all locations (Fig. 3A), whereas the effect of %LOI was more marked at Tablas de Daimiel (Fig. 3B).

During botulism outbreaks, the prevalence of *C. botulinum* type C/D was 18.2% (4/22) in pools of aquatic invertebrates and 33.3% (6/18) in pools of necrophagous invertebrates (Table 2). Positive aquatic invertebrate samples corresponded to two pools of Chironomidae from sediment and two pools of Corixidae from the water column. Positive necrophagous invertebrate samples corresponded to two pools of adults, one pool of pupae, and three pools of larvae of Sarcophagidae and Calliphoridae flies collected from or around bird carcasses.

TABLE 2 Presence of *Clostridium botulinum* in different environmental samples taken from wetlands in Castilla-La Mancha, Spain, between 2005 and 2008

		Presence of *Clostridium botulinum* in environmental samples[a]														
		Sediment			Benthic invertebrates			Water column invertebrates			Waterfowl carcasses			Necrophagous flies		
Wetland	Outbreak yr(s)	No. of samples	No. of positive samples[b]	% positive samples	No. of samples	No. of positive samples[c]	% positive samples	No. of samples	No. of positive samples[d]	% positive samples	No. of samples	No. of positive samples	% positive samples	No. of samples	No. of positive samples[e]	% positive samples
Tablas de Daimiel	2007	93	6	6.5	3	0	0				11	4	36.4			
Navaseca lagoon	2008	14	0	0	7	1	14.3	6	2	33.3	24	7	29.2	10	2	20
Alcázar de San Juan lagoons	2006, 2008	37	4	10.8							29	11	37.9	7	3	42.9
Manjavacas lagoon		9	0	0												
Pedro Muñoz lagoons	2006	15	0	0												
Vega del Jabalon reservoir	2005	21	2	9.5				6	1	16.7	11	7	63.6	1	1	100
Vicario reservoir	2005	18	0	0												
All[f]		207	12	5.8[A]	10	1	10.0[AB]	12	3	25.0[B]	75	29	38.7[B]	18	6	33.3[B]

[a] The number of samples tested, number of *C. botulinum*-positive samples, and percent *C. botulinum*-positive samples are shown for different environmental samples (sediment samples, benthic invertebrates, etc.).

[b] All but two *C. botulinum*-positive samples were collected during botulism outbreaks.

[c] The *C. botulinum*-positive benthic invertebrates belonged to the Chironomidae family.

[d] The *C. botulinum*-positive water column invertebrates belonged to the Corixidae family.

[e] The *C. botulinum*-positive necrophagous flies were adults, pupae, and larvae of members of the Sarcophagidae and Calliphoridae families.

[f] Percentages of the presence of *C. botulinum* were not significantly different between sample types sharing a superscript capital letter.

TABLE 3 Physicochemical characteristics of sediments from wetlands in La Mancha, Spain[a]

Wetland zone	No. of samples	pH		Moisture (%)		Organic matter (%)[b]		PO_4^- (ppm)[b]		NO_3^- (ppm)[b]		NO_2^- (ppm)[b]		Cl^- (ppm)[b]	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Tablas de Daimiel	**182**	**7.68^{AB}**	**0.01**	**58.0^{C}**	**1.0**	**17.9^{B}**	**0.8**	**21.44^{ABC}**	**1.75**	**369.0^{ABC}**	**21.2**	**2.41^{A}**	**0.67**	**1,263^{A}**	**138**
Morenillo	36	7.60	0.03	57.0	3.3	20.3	1.0	39.36	4.69	548.0	83.1	3.34	1.01	1,132	179
Tablazo	70	7.72	0.02	49.8	1.1	8.5	0.3	10.96	1.94	339.8	21.9	3.39	1.65	1,133	193
Exhibit lagoon	1	7.76		57.9		15.1		21.31		279.1		0.48		6,047	
Permanent lagoon	75	7.67	0.02	66.1	0.6	25.7	1.1	22.61	2.54	311.6	20.4	1.07	0.14	1,383	261
Alcázar de San Juan	**30**	**8.31^{C}**	**0.05**	**25.1^{A}**	**2.0**	**12.1^{AB}**	**0.9**	**28.18^{ABC}**	**5.92**	**371.5^{ABC}**	**39.5**	**4.58^{A}**	**1.47**	**34,987^{C}**	**6,058**
La Veguilla	10	8.09	0.08	14.5	1.2	7.7	1.3	21.45	15.86	480.6	84.4	7.64	3.42	3,705	1,568
Las Yeguas	10	8.30	0.09	31.5	1.4	15.1	0.8	27.83	4.25	381.5	57.1	2.04	1.22	66,870	9,335
Camino Villafranca	10	8.54	0.05	29.2	3.9	13.6	1.1	35.26	7.69	252.4	42.5	4.06	2.44	34,386	6,588
Manjavacas	**10**	**8.43^{C}**	**0.06**	**26.8^{A}**	**1.7**	**14.4^{AB}**	**1.6**	**35.42^{BC}**	**10.18**	**482.4^{C}**	**92.3**	**1.65^{A}**	**0.57**	**32,544^{C}**	**4,922**
Pedro Muñoz	**24**	**7.81^{B}**	**0.05**	**38.2^{B}**	**3.3**	**16.5^{B}**	**1.1**	**43.07^{C}**	**12.04**	**427.7^{BC}**	**52.7**	**17.30^{B}**	**6.61**	**13,262^{B}**	**2,220**
Pueblo	14	7.78	0.07	48.3	3.5	18.5	1.5	62.04	19.14	479.7	73.6	19.77	7.24	8,722	2,004
Retamar	10	7.84	0.07	24.1	2.4	13.6	1.1	16.50	3.70	354.8	71.2	13.83	12.62	19,619	3,794
Vega del Jabalón	**20**	**8.50^{C}**	**0.15**	**40.3^{B}**	**3.2**	**7.3^{A}**	**1.5**	**13.16^{AB}**	**6.51**	**183.1^{A}**	**17.2**	**0.36^{A}**	**0.09**	**414^{A}**	**133**
Vicario	**20**	**7.52^{A}**	**0.07**	**44.4^{B}**	**1.9**	**8.3^{A}**	**0.4**	**8.16^{A}**	**2.66**	**249.1^{AB}**	**24.1**	**0.59^{A}**	**0.09**	**1,330^{A}**	**279**

[a] The mean values for each wetland are shown in boldface type. Means sharing a superscript capital letter were not significantly different between wetlands.
[b] These physicochemical characteristics shown on the basis of weight (dry weight).

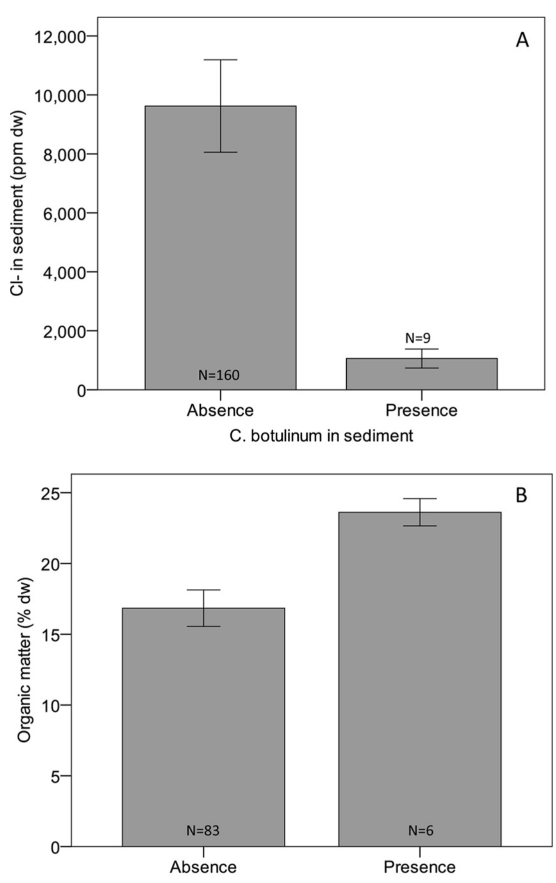

FIG 3 Differences in physicochemical properties of sediments between samples in the absence and presence of *Clostridium botulinum* type C. (A) Concentration of Cl^- (Wald's $\chi^2 = 4.54$; $P = 0.033$). (B) Organic matter content (% loss on ignition) of sediments from Tablas de Daimiel (Wald's $\chi^2 = 5.08$; $P = 0.024$). dw, dry weight.

The presence of *C. botulinum* type C/D was detected in the digestive tract contents of 38.7% of affected birds (Table 2). Within the positive birds, *C. botulinum* was more frequently detected in the cecum samples (16/22 [73%]), followed by cloacal swabs (4/7 [57%]), intestine (14/26 [54%]), and then the gastric content (10/29 [34%]) (see Table S3 in the supplemental material). In positive birds for which several samples were available, detection of *C. botulinum* was more frequent ($P = 0.012$) in cecum samples (15/18 [83%]) than in gastric content samples (5/18 [28%]).

DISCUSSION

The present study shows that outbreaks of avian botulism have been regularly observed over the last 30 years in the wetland area known as "Mancha Húmeda" in Spain, despite there being a relatively low occurrence of *C. botulinum* (5.8%) present in sediment samples. These outbreaks have affected around 20,000 birds from over 50 species (mainly waterfowl, rallids, and waders) and occurred especially when the mean temperature in July was above 26°C. In parallel with the presence of *C. botulinum*, the highest risk of botulism may occur in wetlands with lower salinity (low Cl^- levels) and high organic matter content.

Mouse bioassays performed at the Spanish Institute of Toxicology with hexavalent antitoxin have confirmed the presence of BoNT in outbreaks that occurred between 1998 and 2002. Subsequently, outbreaks that occurred up until 2009 have been confirmed by mouse bioassay at the Instituto de Investigación en Recursos Cinegéticos (IREC), Ciudad Real, Spain, and samples taken during these outbreaks have been determined to be positive both to the C antitoxin (*C. botulinum* antitoxin type C; catalog no. BS0611, lot 05-0100, Centers for Disease Control and Prevention, Atlanta, GA, USA) and to the type D antitoxin in the Institute Pasteur in all cases. In addition, some field samples were positive for the type C/D mosaic which suggests that *C. botulinum* producing avian botulism in Mancha Húmeda, Spain, is probably due to this type (as is the case in other parts of Europe [7]). Negative results using PCR in a few samples that were positive by qPCR can be explained by the lower sensitivity inherent in conventional PCR compared to qPCR, and also because the threshold cycles (C_Ts) in the qPCR were later (data not shown). Further work (I. Anza, H. Skarin, D. Vidal, and R. Mateo, unpublished data) undertaken in collaboration with the National Veterinary Institute in Uppsala, Sweden, also revealed that 13 isolated strains from two outbreaks that occurred in Mancha Húmeda in 2010 were all type C/D and were similar by genotyping to Swedish strains. The present work has been focused on the detection of *C. botulinum* rather than the presence of the neurotoxin. qPCR has proved to be a sensitive tool for the detection of *C. botulinum* in the environment and its ecoepidemiological study.

In general, the wild birds most frequently affected by botulism are waterfowl and shorebirds (2, 3, 42). In our study, 69.3% of the recorded deaths were for the Anatidae family and 12.6% to shorebird species (Scolopacidae, Recurvirostridae, and Charadridae). The most affected species was the mallard duck, which represented 50% of the reported mortality; the same proportion as observed by Woo et al. (27) in an outbreak that occurred in South Korea in 2008. In addition, Shayegani et al. (12) reported high percentages for Anseriformes (56%) and lower percentages for shorebirds (6.6%) in mortalities caused by botulism in New York State. The mallard duck is one of the most abundant species in wetlands in our study area and elsewhere, and therefore, it commonly suffers losses during botulism outbreaks (3, 12). Outbreaks of botulism are an especially significant conservation issue when they affect endangered species with limited local or global distribution. In such cases, isolated population hotspots are highly sensitive to catastrophic events such as unusual weather events or disease outbreaks. For example, a single botulism outbreak produced an estimated die-off of 15 to 20% of the western metapopulation of American white pelican (*Pelecanus erythrorhynchos*) (43), and another in Taiwan caused the death of 73 black-faced spoonbill (*Platalea minor*), while global populations are now estimated at only 1,600 mature individuals (34, 44). In another event, 181 critically endangered Laysan ducks (*Anas laysanensis*) died during a botulism outbreak at Midway Atoll (Hawaii), out of a global population of approximately 500 to 680 mature individuals (33, 34). It should be noted that threatened species, such as the endangered white-headed duck, were also found dead during the botulism outbreaks reported here (see Table S1 in the supplemental material) despite the scarcity of the species in the wetlands studied. Although the population of this species in Spain has increased from less than 100 birds in 1978 to around 2,200 birds in 2007, in Ciudad Real province (where most of our sampling sites were located), the September 2007 census recorded only 139 birds (35). The world population for this species has been estimated to be between just 7,900 and 13,100 individuals (around 5,300 to 8,700 mature individuals [34]) and is in decline. Botulism may therefore be a major concern in terms of conservation efforts aimed at this species.

In principle, an avian botulism outbreak is essentially unpredictable, but it is now known that various environmental factors play a predisposing role in epidemics. These factors include temperature, salinity of the substrate, pH, redox potential (of the surface water and soil/sediment pore water), dissolved oxygen level, and sediment/soil organic matter content (24, 45, 46). Moreover, large epidemic outbreaks are favored by the presence of botulism intoxicated avian carcasses and the maggots that feed on them. This acts to amplify the number of intoxicated birds as they feed on BoNT-bearing maggots in a process known as the carcass-maggot cycle (15). This cycle is favored by temperatures above 20°C which facilitates the growth of *C. botulinum* in the bird carcasses (28, 47). As observed here, the majority of outbreaks recorded in the Mancha Húmeda wetlands in Spain started in July and correlated with mean temperatures above 26°C in this month. The temperature in July frequently reached 40°C during the day within the study period, which is optimal for growing *C. botulinum* type C in an appropriate substrate (47). In this regard, global warming may increase the risk of a botulism outbreak in the area. Natural temporary wetlands would probably be affected by persistent drought, whereas artificial wetlands, such as lagoons receiving treated wastewater, may persist as the only habitat for waterbirds. Rising temperatures may increase the frequency of botulism outbreaks by reducing the number of low-risk saline habitats and favoring the growth of *C. botulinum* itself.

In Castilla-La Mancha, Spain, we have rarely observed botulism outbreaks in spring or autumn, as reported in some wetlands in America (2, 11), Europe (13), and Asia (27). The only recorded case in our data set was an outbreak observed in the Navaseca lagoon, which started at the end of September 2008, when there was a mean temperature of just 20.5°C. The cause of this episode is still unknown. The outbreak began just after a short period of heavy rain, which exceeded the carrying capacity of the wastewater treatment plant that feeds treated water into this lagoon. Further research is necessary to understand the possible epidemiological association between wastewater, enteropathogens, and botulism outbreaks, since many of the botulism epidemics in our study area occurred in wetlands that receive significant inputs from wastewater treatment plants. A feasible hypothesis is that some bacteria of fecal origin could initially cause mortality of a few birds. The onset of several botulism outbreaks has indeed been associated with previous bird mortalities due to other causes, i.e., due to starvation, bacterial or parasitic infections, predation, or pesticide poisoning (27, 28). In turn, this may initiate the carcass-maggot cycle within the optimal environment offered by the decomposing carcass. The likelihood of this happening may be heightened after a high rainfall storm event when relatively untreated sewage may be flushed through the briefly overburdened wastewater treatment plant.

Detection rates for *C. botulinum* type C in the soils/sediments analyzed in our study area (5.8%) were similar to those observed in other affected wetlands in Spain (1.7 to 18% [20, 21]) and Florida (5.6% [18]). However, these values were far lower than in other wetlands in Austria (74 to 83% [48]), California (52% [23]), Canada (38% [19]), or the United Kingdom (19.4 to 51.5% [17]). The marked seasonality of many Mediterranean wetlands may reduce the persistence of *C. botulinum* in sediments. For example, Sandler et al. (23) found differences between the presence of *C. botulinum* in marshes that remained flooded compared with marshes that were drained in the spring and flooded in the fall; they found that prevalence was higher in the permanently flooded marshes. Other factors, such as the high salinity prevailing in most of the La Mancha Húmeda wetlands may also affect the long-term presence of *C. botulinum* in sediments. We have observed a clear negative association between soluble Cl^- levels in sediments and the detection of *C. botulinum*. Likewise, other studies have reported a negative effect of salinity on *C. botulinum* growth (49, 50) and botulism occurrence (2). Further, the highest risk of occurrence for avian botulism outbreaks has been associated with a soil pH value between 7 and 8 (45). We have not observed a significant effect of pH on the presence of *C. botulinum* in the sediments studied here, but most of our observed values were within this cited range of risk. Another important factor (not studied here) is redox potential. Negative values for this parameter, indicative of reducing/low oxygen conditions in the sediment, have been associated with a higher risk of a botulism outbreak (45). The wetlands studied here tended to have a positive redox potential (55), but this may be lower in lagoons fed by wastewater treatment plants or locally near wastewater outfalls as a result of the higher input of fresh organic matter/nutrients (54). Wetlands that receive treated wastewater may therefore pose a higher risk for botulism initiation because they may tend to be more affected by eutrophication (which may induce reducing sedimentary conditions) or provide a substrate (by causing the death of a bird by other means) for toxin production. The continuous unnatural water input/influx may also remove the key annual drought period and reduce salinity compared to other temporary wetlands.

Invertebrates, mainly maggots from necrophagous flies, play a crucial role in botulism outbreaks as vehicles for the BoNT toxin which then causes mortality in the birds feeding on them (15). Hubálek and Halouzka (13) analyzed various invertebrates during a botulism outbreak and detected BoNT at high concentrations in necrophagous larvae and pupae of the blowflies *Lucilia sericata* and *Calliphora vomitoria* which were collected from bird carcasses. In turn, the risk of mortality due to avian botulism tends to be higher in wetlands with higher densities of maggot-laden carcasses (30). Duncan and Jensen (9) compared the toxicity of different invertebrate species collected from or near carcasses and again found that Calliphoridae larvae (collected from carcasses) pose the greatest risk. Toxicity was also analyzed in adult invertebrate forms, and 4 out of 15 samples of Calliphoridae flies were toxin positive. We have detected *C. botulinum* type C/D not only in larvae and pupae but also in adults of Calliphoridae and Sarcophagidae flies. The presence of the bacteria in the adult fly provides a new aspect to be considered in the epidemiology of avian botulism, since these flies may be actively transporting *C. botulinum* type C/D from one carcass to another.

Other invertebrates collected around carcasses, such as ptychopterid fly larvae, leeches, and sow bugs, have been shown to contain BoNT, albeit at concentrations that were lower than in blowfly larvae (9, 13). During mass mortalities of waterbirds produced by *C. botulinum* type E in North America, benthic invertebrates have been identified as potential vectors of spores (51). Here, we detected *C. botulinum* in samples of Chiromonidae and Corixidae that were collected around bird carcasses, but their potential risk as carriers of toxin seems to be limited (9).

The overall prevalence of *C. botulinum* in the digestive tracts of bird carcasses collected during botulism outbreaks was 38.5%, and it was more frequently detected in the lower tract (bowel or cecum) than in the upper tract (gastric content). This difference in distribution may reflect the preference of *C. botulinum* type C/D for the lower tract (especially the cecum) for postmortem growth of the bacteria. Alternatively, it may reflect its premortem prevailing presence. The presence of *C. botulinum* type C in the digestive tracts of birds can be explained by the ingestion of vegetative cells and/or spores (52); furthermore, the bacteria could persist in the ceca of healthy birds where it may act as a substrate for toxin production after death (53). We have noted that cloacal swabs can give similar results to cecum samples in terms of detecting the presence of *C. botulinum* type C/D. This may indicate that cloacal swabs could be the specimen of choice in live birds suspected of having botulism poisoning or could be used to study possible carriers of the microorganism in epidemiological studies.

Overexploitation of groundwater resources in the agricultural land around the Mancha Humeda wetlands in Spain has completely changed the natural water regime in these wetlands. Tablas de Daimiel, which was historically a regionally important permanent wetland, currently undergoes extended periods of drought. In contrast, other historically temporary saline lagoons have now been transformed into permanent freshwater lagoons due to the continuous input provided by wastewater treatment plants. Navaseca is one such artificial lagoon. Our analysis of its physicochemical characteristics indicates that it represents a significant risk in terms of its potential to host/induce local botulism outbreaks which may then be disseminated by vectors to nearby wetlands like Tablas de Daimiel National Park, where the highest numbers of birds concentrate and where the greatest implication for certain endangered avian species may exist.

ACKNOWLEDGMENTS

We thank Victor Diez for information provided regarding botulism outbreaks recorded by the personnel of the JCCM. We also appreciate the cooperation of staff from the Tablas of Daimiel National Park and of the environmental agents from Castilla-La Mancha (JCCM). We also thank Elena de Prada for help with sample collection at the Wildlife Rehabilitation Centre of "El Chaparrillo." Finally, we thank the Department of Bacteriology of the National Veterinary Institute in Uppsala, Sweden, and the Unit of Anaerobic Bacteria and Toxins of the Pasteur Institute in Paris, France, for their receptivity and collaboration during Ibone Anza's two stays at these institutions.

This study was supported by the Spanish Ministry of Environment (grants OAPN 099/2003 and OAPN 035/2009). Dolors Vidal was supported with a JAE DOC contract from the Spanish Council of Research (CSIC). Mark A. Taggart was supported by a Juan de la Cierva Fellowship from the Spanish Ministry of Science and Innovation (MICINN). Ibone Anza was supported by a JAE PRE grant from CSIC, and Elisa Pérez-Ramírez was supported by a I3p grant from CSIC.

REFERENCES

1. **Mitchell WR, Rosendal S.** 1987. Type C botulism: the agent, host susceptibility, and predisposing factors, p 55–71. *In* Eklund W, Dowell VR, Jr (ed), Avian botulism: an international perspective. Charles C Thomas, Publisher, Springfield, IL.
2. **Rocke TE, Friend M.** 1999. Avian botulism, p 271–281. *In* Friend M, Franson JD (ed), Field manual of wildlife disease. General procedures and diseases of birds. US Geological Survey (USGS) National Wildlife Health Center, Washington, DC.
3. **Rocke TE.** 2006. The global importance of avian botulism, p 422–426. *In* Boere GC, Galbraith CA, Stroud DA (ed), Waterbirds around the world. The Stationery Office, Edinburgh, United Kingdom.
4. **Takeda M, Tsukamoto K, Kohda T, Matsui M, Mukamoto M, Kozaki S.** 2005. Characterization of the neurotoxin produced by isolates associated with avian botulism. Avian Dis. **49:**376–381.
5. **Skarin H, Lindberg A, Blomqvist G, Aspán A, Båverud V.** 2010. Molecular characterization and comparison of Clostridium botulinum type C avian strains. Avian Pathol. **39:**511–518.
6. **Lindberg A, Skarin H, Knutsson R, Blomqvist G, Båverud V.** 2010. Real-time PCR for Clostridium botulinum type C neurotoxin (BoNTC) gene, also covering a chimeric C/D sequence–application on outbreaks of botulism in poultry. Vet. Microbiol. **146:**118–123.
7. **Woudstra C, Skarin H, Anniballi F, Fenicia L, Bano L, Drigo I, Koene M, Bäyon-Auboyer MH, Buffereau JP, De Medici D, Fach P.** 2012. Neurotoxin gene profiling of *Clostridium botulinum* types C and D native to different countries within Europe. Appl. Environ. Microbiol. **78:**3120–3127.

8. **Peck MW.** 2009. Biology and genomic analysis of *Clostridium botulinum*. Adv. Microb. Physiol. **55:**183–265.
9. **Duncan RM, Jensen WL.** 1976. A relationship between avian carcasses and living invertebrates in the epizootiology of avian botulism. J. Wildl. Dis. **12:**116–126.
10. **Foreyt WJ, Abinanti FRJ.** 1980. Maggot-associated type C botulism in game farm pheasants. Am. Vet. Med. Assoc. **177:**827–828.
11. **Wobeser G, Rainnie DJ, Smith-Windsor TB, Bogdan G.** 1983. Avian botulism during late autumn and early spring in Saskatchewan. J. Wildl. Dis. **19:**90–94.
12. **Shayegani M, Stone WB, Hannett GE.** 1984. An outbreak of botulism in waterfowl and fly larvae in New York State. J. Wildl. Dis. **20:**86–89.
13. **Hubálek Z, Halouzka J.** 1991. Persistence of *Clostridium botulinum* type C toxin in blow fly (Calliphoridae) larvae as a possible cause of avian botulism in spring. J. Wildl. Dis. **27:**81–85.
14. **Cliplef DJ, Wobeser G.** 1993. Observations on waterfowl carcasses during a botulism epizootic. J. Wildl. Dis. **29:**8–14.
15. **Wobeser G.** 1997. Avian botulism – another perspective. J. Wildl. Dis. **33:**181–186.
16. **Evelsizer DD, Bollinger TK, Dufour KW, Clark RG.** 2010. Survival of radio-marked mallards in relation to management of avian botulism. J. Wildl. Dis. **46:**864–877.
17. **Smith GR, Oliphant JC, White WR.** 1982. *Clostridium botulinum* type C in the Mersey estuary. J. Hyg. (Lond.) **89:**507–511.
18. **Marion WR, O'Meara TE, Riddle GD, Berkhoff HA.** 1983. Prevalence of *Clostridium botulinum* type C in substrates of phosphate-mine settling ponds and implications for epizootics of avian botulism. J. Wildl. Dis. **19:**302–307.
19. **Wobeser G, Marsden S, MacFarlane RJ.** 1987. Occurrence of toxigenic *Clostridium botulinum* type C in the soil of wetlands in Saskatchewan. J. Wildl. Dis. **23:**67–76.
20. **Contreras de Vera A, García Fernandez A, Cubero Pablo MJ, León-Vizcaíno L.** 1991. Low prevalence of *C. botulinum* in Spanish wetlands with a tidal regime. Vet. Rec. **128:**187–188.
21. **Contreras de Vera A, León-Vizcaíno L, Carranza Guzmán J, Hermoso de Mendoza M, Garrido Abellán F.** 1987. Botulismo aviar en zonas húmedas de la campiña de Sevilla y Córdoba. Med. Vet. **4:**173–179.
22. **Villalba EJ, Haagsma J, Díaz EJ, Gasca A.** 1989. Niveles de contaminación por *Clostridium botulinum* y sus toxinas en diferentes hidrohábitats del Parque Nacional de Doñana. Med. Vet. **6:**559–561.
23. **Sandler RJ, Rocke TE, Samuel MD, Yuill TM.** 1993. Seasonal prevalence of *Clostridium botulinum* type C in sediments of a northern California wetland. J. Wildl. Dis. **29:**533–539.
24. **Babinszky G, Csitári C, Józsa S.** 2008. Observations on environmental factors in connection with avian botulism outbreaks in a Hungarian wetland habitat. Acta Microbiol. Immunol. Hung. **55:**455–464.
25. **Borland ED, Moryson CJ, Smith GR.** 1977. Avian botulism and the high prevalence of *Clostridium botulinum* in the Norflok Broads. Vet. Rec. **100:**106–109.
26. **Wobeser G, Galmut EA.** 1984. Internal temperature of decomposing duck carcasses in relation to botulism. J. Wildl. Dis. **20:**267–271.
27. **Woo GH, Kim HY, Bae YC, Jean YH, Yoon SS, Bak EJ, Hwang EK, Joo YS.** 2010. Outbreak of botulism (*Clostridium botulinum* type C) in wild waterfowl: Seoul, Korea. J. Wildl. Dis. **46:**951–955.
28. **Soos C, Wobeser G.** 2006. Identification of primary substrate in the initiation of avian botulism outbreaks. J. Wildl. Manag. **70:**43–53.
29. **Crauste F, Hbid ML, Kacha A.** 2008. A delay reaction-diffusion model of the dynamics of botulinum in fish. Math. Biosci. **216:**17–29.
30. **Evelsizer DD, Clark RG, Bollinger TK.** 2010. Relationships between local carcass density and risk of mortality in molting mallards during avian botulism outbreaks. J. Wildl. Dis. **46:**507–513.
31. **Dohms JE, Cloud SS.** 1982. Susceptibility of broiler chickens to *Clostridium botulinum* type C toxin. Avian Dis. **26:**89–96.
32. **Wobeser G.** 1988. Effects of botulism on ducks drinking saline water. J. Wildl. Dis. **24:**240–245.
33. **Work TM, Klavitter JL, Reynolds MH, Blehert D.** 2010. Avian botulism: a case study in translocated endangered Laysan ducks (*Anas laysanensis*) on Midway Atoll. J. Wildl. Dis. **46:**499–506.
34. **International Union for Conservation of Nature and Natural Resources (IUCN).** 2012. The IUCN Red List of Threatened Species (ver. 2012.1). IUCN Global Species Programme Red List Unit, Cambridge, United Kingdom. http://www.iucnredlist.org. Accessed 12 March 2013.
35. **Torres-Esquivias JA.** 2008. La malvasía cabeciblanca, p 54–69. *In* Ballesteros G, Cabrera M, Echevarrías JL, Lorenzo JA, Raya C, Torres-Esquivias JA, Viedma C (ed), Tarro Canelo, cerceta pardilla, porrón pardo, malvasía cabeciblanca y focha moruna en España. Población en 2007 y método de censo. SEO/BirdLife, Madrid, Spain.
36. **León-Quinto T, de la Vega A, Lozano A, Pastor S.** 2004. Summer mortality of waterbirds in a Mediterranean wetland. Waterbirds **27:**46–53.
37. **Vidal D, Taggart MA, Badiola I, Mateo R.** 2011. Real-time polymerase chain reaction for the detection of toxigenic *Clostridium botulinum* type C1 in waterbird and sediment samples: comparison with other PCR techniques. J. Vet. Diagn. Invest. **23:**942–946.
38. **Torres-Esquivias JA.** 2004. Malvasía cabeciblanca, Oxyura leucocephala. *In* Madroño A, González C, Atienza JC (ed), Libro rojo de las aves de España. Dirección General para La Biodiversidad-SEO/BirdLife, Madrid, Spain.
39. **Álvarez-Cobelas M, Cirujano S.** 1996. Las Tablas de Daimiel. Ecología Acuática y Sociedad. Organismo Autónomo de Parques Nacionales, Ministerio de Medio Ambiente, Madrid, Spain.
40. **Cirujano S, Medina L.** 2002. Plantas acuáticas de las lagunas y humedales de Castilla-la Mancha. *In* Cirujano S, Medina L. (ed), Real Jardín Botánico. CSIC and Junta de Comunidades de Castilla-La Mancha, Madrid, Spain.
41. **Sánchez-Hernández L, Cifuentes A, Jiménez B, Mateo R, González R.** 2008. Detection of *Clostridium botulinum* neurotoxin coding genes: analysis of PCR products by real time versus capillary gel electrophoresis methods. Eur. Food Res. Technol. **227:**495–502.
42. **Friend M.** 2002. Avian disease at the Salton Sea. Hydrobiologia **473:**293–306.
43. **Rocke T, Converse K, Meteyer C, Mclean RG.** 2005. The impact of disease in the American white pelican in North America. Waterbirds **28**(Special Publication 1):87–94.
44. **Chuang WC, Shieh YC, Chen SH, Lee YF, Tsai KY, Tsai SS.** 2005. Outbreaks of botulism due to type C1 toxin in black-faced spoonbills (*Platalea minor*). Taiwan Vet. J. **31:**267–273.
45. **Rocke TE, Samuel MD.** 1999. Water and sediment characteristics associated with avian botulism outbreaks in wetlands. J. Wildl. Manag. **63:**1249–1260.
46. **Murphy T, Lawson A, Nalewajko C, Murkin H, Ross L, Oguma K, McIntyre T.** 2000. Algal toxins – initiators of avian botulism? Environ. Toxicol. **15:**558–567.
47. **Rocke TE, Bollinger TK.** 2007. Avian botulism, p 377–416. *In* Thomas NJ, Hunter DB, Atkinson CT (ed), Infectious diseases of wild birds. Wiley-Blackwell, Ames, IA.
48. **Zechmeister TC, Kirschner AK, Fuchsberger M, Gruber SG, Suess B, Rosengarten R, Pittner F, Mach RL, Herzig A, Farnleitner AH.** 2005. Prevalence of botulinum neurotoxin C1 and its corresponding gene in environmental samples from low and high risk avian botulism areas. ALTEX **22:**185–195.
49. **Segner WP, Schmidt CF, Boltz JK.** 1971. Enrichment, isolation, and cultural characteristics of marine strains of *Clostridium botulinum* type C. Appl. Microbiol. **22:**1017–1024.
50. **Webb MD, Pin C, Peck MW, Stringer SC.** 2007. Historical and contemporary NaCl concentrations affect the duration and distribution of lag times from individual spores of nonproteolytic *Clostridium botulinum*. Appl. Environ. Microbiol. **73:**2118–2127.
51. **Pérez-Fuentetaja A, Clapsadl MD, Getchell RG, Bowser PR, Lee WT.** 2011. *Clostridium botulinum* type E in Lake Erie: inter-annual differences and role of benthic invertebrates. J. Great Lakes Res. **37:**238–244.
52. **Haagsma J.** 1991. Pathogenic anaerobic bacteria and the environment. Rev. Sci. Tech. **10:**749–764.
53. **Zhu XY, Zhong T, Pandya Y, Joerger RD.** 2002. 16S rRNA-based analysis of microbiota from the cecum of broiler chickens. Appl. Environ. Microbiol. **68:**124–137.
54. **Hijosa-Valsero M, Sidrach-Cardona R, Bécares E.** 2012. Comparison of interannual removal variation of various constructed wetland types. Sci. Total Environ. **430:**174–183.
55. **Anza I, Vidal D, Sánchez-Prieto S, Díaz-Sánchez S, Martínez C, Laguna C, Chicote A, Gosálvez RU, Florín M, Mateo R.** Study of the sanitary risks for waterbirds associated to the inputs of treated wastewater into the Tablas of Daimiel National Park and nearby wetlands. *In* Ramirez L, Asensio B (ed), Proyectos de Investigación en Parques Nacionales: 2009–2012, in press. Ministerio de Medio Ambiente, Madrid, Spain. (In Spanish.)